

FORM PTO-1590 (REV. 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 7250-12	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>09/889715</b>	
INTERNATIONAL APPLICATION NO. PCT/GB00/00145		INTERNATIONAL FILING DATE January 21, 2000		PRIORITY DATE CLAIMED January 21, 1999	
TITLE OF INVENTION CELL GROWTH					
APPLICANT(S) FOR DO/EO/US Douglas W. Hamilton; Christopher L. Ives; Ian P. Middleton; Chiara Rossetto					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input checked="" type="checkbox"/> has been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> is attached hereto.</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p style="margin-left: 20px;">d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). <i>Unsigned</i></p> <p>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p>					
Items 11 to 20 below concern document(s) or information included:					
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.					
12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.					
13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.					
14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.					
15. <input type="checkbox"/> A substitute specification.					
16. <input type="checkbox"/> A change of power of attorney and/or address letter.					
17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.					
18. <input checked="" type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).					
19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).					
20. <input checked="" type="checkbox"/> Other items or information: International Preliminary Examination Report					

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I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail" service under 37 CFR 1.10 on the date above and is addressed to the Assistant Commissioner, Washington, D.C. 20231.

*Gail Mercer*

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U.S. APPLICATION NO. (if known) 09/889715

INTERNATIONAL APPLICATION NO.  
PCT/GB00/00145ATTORNEY'S DOCKET NUMBER  
7250-1221. ☒ The following fees are submitted:**BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):**Neither international preliminary examination fee (37 CFR 1.482)  
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO  
and International Search Report not prepared by the EPO or JPO..... \$1000.00International preliminary examination fee (37 CFR 1.482) not paid to  
USPTO but International Search Report prepared by the EPO or JPO ..... \$860.00International preliminary examination fee (37 CFR 1.482) not paid to USPTO  
but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$710.00International preliminary examination fee (37 CFR 1.482) paid to USPTO  
but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$690.00International preliminary examination fee (37 CFR 1.482) paid to USPTO  
and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00**ENTER APPROPRIATE BASIC FEE AMOUNT =****CALCULATIONS PTO USE ONLY**

\$ 860.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30  
months from the earliest claimed priority date (37 CFR 1.492(e)).

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$
Total claims	65 - 20 =	45	x \$18.00	\$ 810.00
Independent claims	5 - 3 =	2	x \$80.00	\$ 160.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$270.00	\$
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$ 1830.00
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$ 915.00
<b>SUBTOTAL =</b>				\$ 915.00
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$
<b>TOTAL NATIONAL FEE =</b>				\$ 915.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$
<b>TOTAL FEES ENCLOSED =</b>				\$ 915.00
				Amount to be refunded: \$
				charged: \$

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

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NAME

28,309

REGISTRATION NUMBER

09/889715

JC18 Rec'd PCT/PTO 20 JUL 2001

PBA/NE/D088342PUS:TQH:135657

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of: ) Express Mail No. EL683236140US  
) July 20, 2001  
Douglas William Hamilton et al. )  
)  
Serial No. (unknown) )  
)  
Filed Herewith )  
)  
CELL GROWTH )  
)  
US National Stage of PCT/GB00/00145 )  
International Filing Date January 21, 2000 )

**PRELIMINARY AMENDMENT**

Hon. Assistant Commissioner of Patents

Washington, D.C. 20231

Sir:

Please enter the following Preliminary Amendment in the above-identified patent application. The Commissioner is hereby authorized to charge payment of any additional fees associated with this application or credit any overpayment to Deposit Account No. 23-3030.

**IN THE CLAIMS**

Please amend the claims to read as follows:

"Express Mail" label number EL683236140US. Date of Deposit July 20, 2001  
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR § 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Trademarks, 2900 Crystal Dr., Arlington, Virginia 22202-3513.  
Gail Mercer  
Signature of Person Mailing Correspondence

## Clean Copy of Amended Claims

### Claims

1. A substrate for cell growth comprising a polysaccharide and a cell adhesion protein provided at the surface of the substrate and non-covalently associated therewith.
2. A substrate as claimed in claim 1 comprising a polysaccharide basal layer having a surface layer incorporating the cell adhesion protein.
3. A substrate as claimed in claim 2 wherein the polysaccharide basal layer has a thickness greater than 60% of the thickness of this layer and the polysaccharide basal layer.
4. A substrate as claimed in claim 3 wherein the polysaccharide basal layer has a thickness greater than 80% of the thickness of this layer and the polysaccharide basal layer.
5. A substrate as claimed in claim 2 wherein the cell adhesion protein layer is integral with the polysaccharide basal layer.
6. A substrate as claimed in claim 5 wherein the layer of the cell adhesion protein has a thickness of 1-20 $\mu$ m.
7. A substrate as claimed in claim 2 wherein the layer of the cell adhesion protein is a surface adsorbed layer.
8. A substrate as claimed in claim 7 wherein the layer of the cell adhesion protein is 3-5 molecules deep.
9. A substrate as claimed in claim 2 wherein the polysaccharide basal layer comprises at least 80% by weight of polysaccharide.

10. A substrate as claimed in claim 9 wherein the polysaccharide basal layer comprises at least 90% by weight of polysaccharide.
11. A substrate as claimed in claim 2 wherein the surface layer of the cell adhesion protein comprises at least 80% by weight of cell adhesion protein.
12. A substrate as claimed in claim 11 wherein the surface layer of the cell adhesion protein comprises at least 90% by weight of cell adhesion protein.
13. A substrate as claimed in claim 12 wherein the surface layer of cell adhesion protein comprises 95 to 100% by weight of cell adhesion protein.
14. A substrate as claimed in claim 2 wherein the cell adhesion protein layer is discontinuous layer.
15. A substrate as claimed in claim 2 wherein the polysaccharide layer incorporates an active agent.
16. A substrate as claimed in claim 15 wherein the active agent is encapsulated.
17. A substrate as claimed in claim 15 wherein the active agent is a drug, growth factor or chemotactic agent.
18. A substrate as claimed in claim 1 wherein the polysaccharide is selected from alginates, chitosan, polylysine and other polyamino acids, cationic starches, cationic derivatives of other hydrocolloids, collagen, gelatine, low-methoxyl pectin, carrageenans, chondroitin sulphate, hyaluronic acid, carboxymethyl cellulose, carboxymethyl starch, carboxymethyl guar, cellulose sulphate, dextran sulphate, gellan, xanthan, and anionic derivatives of other hydrocolloids.
19. A substrate as claimed in claim 18 wherein the polysaccharide is an alginate.

20. A substrate as claimed in claim 19 wherein the alginate has a Guluronic acid (G) content of at least 35% by weight and Mannuronic acid (M) content of at most 65% by weight.

21. A substrate as claimed in claim 20 wherein the polysaccharide as a G content of 35-70% by weight and an M content of 65-30% by weight.

22. A substrate as claimed in claim 19 wherein the alginate is cross-linked with divalent cations, preferably calcium ions.

23. A substrate as claimed in claim 22 wherein the cell adhesion protein is stabilised by calcium ion bridges.

24. A substrate as claimed in claim 18 wherein the polysaccharide is chitosan.

25. A substrate as claimed in claim 24 wherein the chitosan comprises at least 70% de-acetylated chitin.

26. A substrate as claimed in claim 1 wherein the cell adhesion protein is present in blood plasma.

27. A substrate as claimed in claim 1 wherein the cell adhesion protein incorporates the RGD binding site.

28. A substrate as claimed in claim 27 wherein the cell adhesion protein is fibronectin, vitronectin or von Willebrand protein.

29. A substrate as claimed in claim 28 wherein the cell adhesion protein is fibronectin.

30. A substrate for cell growth comprising a polysaccharide and a blood plasma component provided at the surface of the substrate and non-covalently associated therewith.
31. A substrate as claimed in claim 1 in the form of a fibre.
32. A substrate as claimed in claim 31 wherein the fibre has a diameter of 10-1000 $\mu$ m.
33. A substrate as claimed in claim 32 wherein the fibre has a diameter of 40-150 $\mu$ m.
34. A substrate as claimed in claim 33 wherein the fibre has a diameter of 40-100 $\mu$ m.
35. A substrate as claimed in claim 34 wherein the fibre has a diameter of 50-80 $\mu$ m.
36. A substrate as claimed in claim 1 which is in the form of a sheet or film.
37. A substrate as claimed in claim 36 having a thickness of 2-2000 $\mu$ m.
38. A substrate as claimed in claim 37 having a thickness of 10-100 $\mu$ m.
39. A substrate as claimed in claim 37 having a thickness of 200-1000 $\mu$ m.
40. A substrate as claimed in claim 37 having a thickness of 500-2000 $\mu$ m.
41. An assembly of fibres as claimed in claim 31.
42. An assembly as claimed in claim 41 in the form of a random matrix (e.g. a non-woven felt or fleece), orientated matrix (fibres having some relative alignment), a knitted structure, a braided structure, a bundled structure or a carded sliver.
43. An assembly comprising a plurality of fibres as claimed in claim 31 wherein the fibres are arranged in parallel to each other.

44. An assembly comprising a plurality of fibres as claimed in claim 31 wherein the fibres are arranged randomly.

45. An assembly as claimed in claim 43 wherein the fibres are provided on a support in the form of a sheet or film.

46. An assembly as claimed in claim 45 wherein the fibres are provided on a high MVTR film.

47. An assembly as claimed in claim 41 wherein said fibres are provided in a matrix of an amorphous gel.

48. A method of producing a cell growth substrate comprising extruding a solution containing dissolved polysaccharide and cell adhesion protein, the polysaccharide being present in an amount greater than the cell adhesion protein, into a coagulation bath such that there is precipitated a substrate comprised of a basal layer of a polysaccharide and a surface layer of cell adhesion protein non-covalently associated therewith.

49. A method as claimed in claim 48 wherein the substrate is precipitated in a bath containing divalent cations typically calcium ions.

50. A method as claimed in claim 48 wherein the solution to be extruded comprises (based on the total weight of the polysaccharide and cell adhesion protein) 60-99% by weight of the polysaccharide and 1-40% by weight of the cell adhesion protein.

51. A method as claimed in claim 49 wherein the dissolved polysaccharide is sodium alginate.

52. A method as claimed in claim 51 wherein the sodium alginate has a G-content of 35-70% by weight and an M-content of 65-35% by weight.



53. A method as claimed in claim 51 wherein the sodium alginate has a viscosity for a 1% solution (in water) of 30-300 cP.

54. A method as claimed in claim 53 wherein the sodium alginate has a viscosity of a 1% solution (in water) of 40-100 cP.

55. A method of producing a cell growth substrate comprising applying to the surface of a preformed layer of a polysaccharide a surface layer of a cell adhesion protein or blood plasma component such that said cell adhesion protein or blood plasma component is non-covalently associated with the polysaccharide.

56. A method as claimed in claim 55 wherein the method of application is by immersion of the polysaccharide layer in a coating bath containing the cell adhesion protein or blood plasma component.

57. A method as claimed in claim 55 wherein the polysaccharide is precipitated into a coagulation bath containing the cell adhesion protein.

58. A method as claimed in claim 55 wherein the method of application is by spraying.

59. A method as claimed in claim 55 effected with stabilisation of the protein layer.

60. A method of cell culture comprising effecting growth of cells on a substrate as claimed in claim 1.

61. A method of cell culture comprising effecting growth of cells on an assembly as claimed in claim 41.

62. A method as claimed in 60 wherein the cell growth is for wound repair, tissue repair or augmentation, culturing epidermal or dermal substitute, orthopaedic application, vascular grafts, nerve regeneration or cell culture in a bioreactor.

63. A method as claimed in 61 wherein the cell growth is for wound repair, tissue repair or augmentation, culturing epidermal or dermal substitute, orthopaedic application, vascular grafts, nerve regeneration or cell culture in a bioreactor.

64. The use of a substrate as claimed in claim 1 .

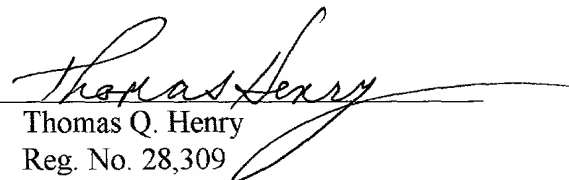
65. The use of an assembly as claimed in claim 41 in therapy.

**REMARKS**

Consideration and allowance of the above-identified patent application is requested.

Respectfully submitted,

By:



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Version with Markings to Show Changes MadeClaims

1. A substrate for cell growth comprising a polysaccharide and a cell adhesion protein provided at the surface of the substrate and non-covalently associated therewith.
2. A substrate as claimed in claim 1 comprising a polysaccharide basal layer having a surface layer incorporating the cell adhesion protein.
3. A substrate as claimed in claim 2 wherein the polysaccharide basal layer has a thickness greater than 60% of the thickness of this layer and the polysaccharide basal layer.
4. A substrate as claimed in claim 3 wherein the polysaccharide basal layer has a thickness greater than 80% of the thickness of this layer and the polysaccharide basal layer.
5. A substrate as claimed in ~~any one of claims 2 to 4~~ wherein the cell adhesion protein layer is integral with the polysaccharide basal layer.
6. A substrate as claimed in claim 5 wherein the layer of the cell adhesion protein has a thickness of 1-20 $\mu$ m.
7. A substrate as claimed in ~~any one of claims 2 to 5~~ wherein the layer of the cell adhesion protein is a surface adsorbed layer.
8. A substrate as claimed in claim 7 wherein the layer of the cell adhesion protein is 3-5 molecules deep.
9. A substrate as claimed in ~~any one of claims 2 to 8~~ wherein the polysaccharide basal layer comprises at least 80% by weight of polysaccharide.
10. A substrate as claimed in claim 9 wherein the polysaccharide basal layer comprises at least 90% by weight of polysaccharide.

11. A substrate as claimed in ~~any one of claims 2 to 10~~ wherein the surface layer of the cell adhesion protein comprises at least 80% by weight of cell adhesion protein.
12. A substrate as claimed in claim 11 wherein the surface layer of the cell adhesion protein comprises at least 90% by weight of cell adhesion protein.
13. A substrate as claimed in claim 12 wherein the surface layer of cell adhesion protein comprises 95 to 100% by weight of cell adhesion protein.
14. A substrate as claimed in ~~any one of claims 2 to 13~~ wherein the cell adhesion protein layer is discontinuous layer.
15. A substrate as claimed in ~~any one of claims 2 to 14~~ wherein the polysaccharide layer incorporates an active agent.
16. A substrate as claimed in claim 15 wherein the active agent is encapsulated.
17. A substrate as claimed in claim 15 ~~or 16~~ wherein the active agent is a drug, growth factor or chemotactic agent.
18. A substrate as claimed ~~in any one of claims 1 to 17~~ wherein the polysaccharide is selected from alginates, chitosan, polylysine and other polyamino acids, cationic starches, cationic derivatives of other hydrocolloids, collagen, gelatine, low-methoxyl pectin, carrageenans, chondroitin sulphate, hyaluronic acid, carboxymethyl cellulose, carboxymethyl starch, carboxymethyl guar, cellulose sulphate, dextran sulphate, gellan, xanthan, and anionic derivatives of other hydrocolloids.
19. A substrate as claimed in claim 18 wherein the polysaccharide is an alginate.
20. A substrate as claimed in claim 19 wherein the alginate has a Guluronic acid (G) content of at least 35% by weight and Mannuronic acid (M) content of at most 65% by weight.

21. A substrate as claimed in claim 20 wherein the polysaccharide as a G content of 35-70% by weight and an M content of 65-30% by weight.
22. A substrate as claimed in ~~any one of claims 19 to 21~~ wherein the alginate is cross-linked with divalent cations, preferably calcium ions.
23. A substrate as claimed in claim 22 wherein the cell adhesion protein is stabilised by calcium ion bridges.
24. A substrate as claimed in claim 18 wherein the polysaccharide is chitosan.
25. A substrate as claimed in claim 24 wherein the chitosan comprises at least 70% de-acetylated chitin.
26. A substrate as claimed in ~~any one of claims 1 to 25~~ wherein the cell adhesion protein is present in blood plasma.
27. A substrate as claimed in ~~any one of claims 1 to 26~~ wherein the cell adhesion protein incorporates the RGD binding site.
28. A substrate as claimed in claim 27 wherein the cell adhesion protein is fibronectin, vitronectin or von Willebrand protein.
29. A substrate as claimed in claim 28 wherein the cell adhesion protein is fibronectin.
30. A substrate for cell growth comprising a polysaccharide and a blood plasma component provided at the surface of the substrate and non-covalently associated therewith.
31. A substrate as claimed in ~~any one of claims 1 to 30~~ in the form of a fibre.

32. A substrate as claimed in claim 31 wherein the fibre has a diameter of 10-1000 $\mu$ m.
33. A substrate as claimed in claim 32 wherein the fibre has a diameter of 40-150 $\mu$ m.
34. A substrate as claimed in claim 33 wherein the fibre has a diameter of 40-100 $\mu$ m.
35. A substrate as claimed in claim 34 wherein the fibre has a diameter of 50-80 $\mu$ m.
36. A substrate as claimed in ~~any one of claims 1 to 30~~ which is in the form of a sheet or film.
37. A substrate as claimed in claim 36 having a thickness of 2-2000 $\mu$ m.
38. A substrate as claimed in claim 37 having a thickness of 10-100 $\mu$ m.
39. A substrate as claimed in claim 37 having a thickness of 200-1000 $\mu$ m.
40. A substrate as claimed in claim 37 having a thickness of 500-2000 $\mu$ m.
41. An assembly of fibres as claimed in ~~any one of claims 31 to 35~~.
42. An assembly as claimed in claim 41 in the form of a random matrix (e.g. a non-woven felt or fleece), orientated matrix (fibres having some relative alignment), a knitted structure, a braided structure, a bundled structure or a carded sliver.
43. An assembly comprising a plurality of fibres as claimed in ~~any one of claims 31 or 35~~ wherein the fibres are arranged in parallel to each other.
44. An assembly comprising a plurality of fibres as claimed in ~~any one of claims 31 or 34~~ wherein the fibres are arranged randomly.

45. An assembly as claimed in claim 43 ~~or 44~~ wherein the fibres are provided on a support in the form of a sheet or film.

46. An assembly as claimed in claim 45 wherein the fibres are provided on a high MVTR film.

47. An assembly as claimed in claim 41 wherein said fibres are provided in a matrix of an amorphous gel.

48. A method of producing a cell growth substrate comprising extruding a solution containing dissolved polysaccharide and cell adhesion protein, the polysaccharide being present in an amount greater than the cell adhesion protein, into a coagulation bath such that there is precipitated a substrate comprised of a basal layer of a polysaccharide and a surface layer of cell adhesion protein non-covalently associated therewith.

49. A method as claimed in claim 48 wherein the substrate is precipitated in a bath containing divalent cations typically calcium ions.

50. A method as claimed in claim 48 ~~or 49~~ wherein the solution to be extruded comprises (based on the total weight of the polysaccharide and cell adhesion protein) 60-99% by weight of the polysaccharide and 1-40% by weight of the cell adhesion protein.

51. A method as claimed in claim 49 ~~or 50~~ wherein the dissolved polysaccharide is sodium alginate.

52. A method as claimed in claim 51 wherein the sodium alginate has a G-content of 35-70% by weight and an M-content of 65-35% by weight.

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53. A method as claimed in claim 51 ~~or 52~~ wherein the sodium alginate has a viscosity for a 1% solution (in water) of 30-300 cP.

54. A method as claimed in claim 53 wherein the sodium alginate has a viscosity of a 1% solution (in water) of 40-100 cP.

55. A method of producing a cell growth substrate comprising applying to the surface of a preformed layer of a polysaccharide a surface layer of a cell adhesion protein or blood plasma component such that said cell adhesion protein or blood plasma component is non-covalently associated with the polysaccharide.

56. A method as claimed in claim 55 wherein the method of application is by immersion of the polysaccharide layer in a coating bath containing the cell adhesion protein or blood plasma component.

57. A method as claimed in claim 55 wherein the polysaccharide is precipitated into a coagulation bath containing the cell adhesion protein.

58. A method as claimed in claim 55 wherein the method of application is by spraying.

59. A method as claimed in ~~any one of claims 55 to 58~~ effected with stabilisation of the protein layer.

60. A method of cell culture comprising effecting growth of cells on a substrate as claimed in ~~any one of claims 1 to 40 or an assembly as claimed in any one of claims 41 to 47.~~

61. A method of cell culture comprising effecting growth of cells on an assembly as claimed in claim 41.

63. A method as claimed in 61 wherein the cell growth is for wound repair, tissue repair or augmentation, culturing epidermal or dermal substitute, orthopaedic application, vascular grafts, nerve regeneration or cell culture in a bioreactor.

~~624. The use of a substrate as claimed in any one of claims 1 to 40 or an assembly as claimed in any one of claims 41 to 47 in therapy.~~

65. The use of an assembly as claimed in claim 41 in therapy.

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CELL GROWTH

The present invention relates to substrates for use in cell growth and to methods of producing such substrates. The invention relates more particularly to substrates having cell adhesion promoting activity which may be used in various cell growth applications, e.g. wound healing and tissue engineering. The invention also relates to methods of preparing such substrates and their use in various cell growth applications.

All eukaryotic, mammalian cells are substrate dependent in that they need to be attached to a surface in order to be able to grow, or secrete or divide. The phenotype that cells express is partly determined by their interaction with the substrate to which they are attached. The substrate to which mammalian cells are attached is collagen. All body soft (excluding blood) and hard tissues are made up of cells attached to a framework of collagen. Collagen is a protein that forms fibres and the fibres form matrices, these matrices may form any configuration from random to aligned.

The collagen fibres are themselves made up of fibrils so a collagen fibre resembles a cable of aligned fibrils. The chemistry of the collagen fibril varies according to the tissue type and a range of collagens have been identified.

Substrates for tissue augmentation or to act as carriers for cultured cell transfer in wound therapy are usually collagen based. In this situation, the collagen substrate usually has to be specific to the type of cell growth required and the phenotype and status (secretory, replicatory) grown on the substrate may not turn out to be as required.

US-A-5 610 148 (R.Brown) entitled "Macroscopically Orientated Cell Adhesion Protein" describes the production of a fibre comprised of fibrils of a cell adhesion protein selected from fibronectin (Fn), vitronectin and von Willebrand protein that has been denatured and the polymer chains then aligned by unidirectional

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shear allowing aggregation and precipitation. These fibres are of a fibular construction not dissimilar in some respects to collagen. Cells seeded onto the fibres demonstrate directional cell growth as a result of the longitudinal orientation of the cell adhesion binding site. However such fibre structures require a high concentration of fibronectin or fibrinogen/fibronectin, are somewhat complicated to produce and are of relatively low strength.

It is an object of the present invention obviate or mitigate the above mentioned disadvantages.

According to the present invention there is provided a substrate for cell growth comprising a polysaccharide and a cell adhesion protein provided at the surface of the substrate.

Substrates in accordance with the invention have the advantage (over substrates comprised of fibrils of fibronectin or other cell adhesion protein) of being of higher strength than a substrate comprised substantially of 100% protein and are also easier to manufacture. The substrates of the invention may be used in a wide range of cell growth applications, e.g. wound repair, tissue repair or augmentation, or for the growth of cells in routine cell culture in vitro, in large scale cell culture, bioreactors or organ culture.

In the substrates of the invention, the orientation of the cell adhesion protein is not necessarily significant and guidance of the cells during growth thereof is achieved by the physical form of the substrate. Thus, for example, in the case of a fibre (see below) cell growth may occur along and/or around the fibre as determined by the presence of the cell adhesion protein. We do not however preclude the possibility of the cell adhesion protein having at least some degree of alignment.

The cell adhesion protein preferably incorporates the RGD (Arginine, Glycine, Aspartic acid) binding site. It is particularly preferred that the cell adhesion

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protein is fibronectin, vitronectin or von Willebrand protein or a fragment of such proteins incorporating this RGD binding site.

The preferred cell adhesion protein is fibronectin which may be used in the form routinely isolated from blood plasma, e.g. by cryoprecipitation. The fibronectin may contain fibrinogen and albumin.

The polysaccharide and the cell adhesion protein may be uniformly distributed throughout the substrate so that the cell adhesion protein is present at the surface as a result of this distribution.

The substrate may comprise a polysaccharide basal layer having a surface layer of a cell adhesion protein.

The polysaccharide basal layer will for preference have a thickness of at least 60%, more preferably at least 80% and ideally at least 90% of the combined depth of the basal layer and cell adhesion protein layer.

The cell adhesion protein provided as a surface layer for the polysaccharide basal layer may be an integral layer or may be a surface absorbed molecular layer. The surface layer of the cell adhesion protein may, depending on the method by which it is produced, be only several molecules thick or may be of somewhat greater thickness so as to form a discrete outer layer. Thus, the protein layer may be anything from 3-5 molecules "deep" in the case of surface adsorption to, say, 20 $\mu$ m (e.g. 1-20  $\mu$ m) when formed as a "coating". This protein layer may be an essentially amorphous network, have some crystallinity or even little or no fibril structure. The protein layer may be stabilised and attached to the basal (polysaccharide) layer to different degrees by different physical and/or chemical mechanisms. Examples of such attachment and stabilisation including covalent bonding, hydrogen bonding, van der Waals forces and physical entrapment. In the case where the polysaccharide incorporate carboxylic groups, covalent attachment may be achieved by a carbodiimide which "couples" a carboxylic group of the polysaccharide with an amino group of a protein. A further

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possibility is the use of a melamine-formaldehyde resin. The degree of stability of the protein layer can be used as a mechanism to drive certain cell responses. Thus the substrate may be "tailored" to ensure growth of a particular cell type and/or to provide a known degree of cell growth in a predetermined time.

The polysaccharide layer will for preference comprise at least 50%, more preferably at least 60%, even more preferably at least 80% and ideally at least 90% polysaccharide. The cell adhesion protein layer will preferably comprise at least 50%, more preferably at least 60% even more preferably at least 80% and ideally at least 90% of cell adhesion protein.

The cell adhesion protein layer may incorporate proteins other than cell adhesion proteins.

Cell growth substrates in accordance with the invention may incorporate, e.g. in the polysaccharide layer, an active agent for delivery during the cell growth application. This agent may, for example, be deliverable by diffusion and might for example be a drug. Further examples of active agents include growth factors, chemotactic agents etc. The active agents may be free or encapsulated, for example in lipid type droplets. The active agent may be disposed continuously or discontinuously along, across and/or around the cell growth substrate and may be provided in different amounts at different regions of the substrate so as to establish a concentration gradient.

Substrates in accordance with the invention may be produced by a number of methods. In one such method, a solution containing dissolved polysaccharide and cell adhesion protein (the solution containing less of the protein than the polysaccharide) is extruded into a coagulation bath. We believe that, in such a method, there is preferential deposition of the cell adhesion. The coagulation bath may incorporate, for example, di- or higher- valent cations (e.g.  $\text{Ca}^{2+}$ ) which serve to effect the precipitation and also stabilise the protein layer by ion bridging.

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In a further method, the polysaccharide is extended into a coagulation both which incorporates protein (containing the cell adhesion protein) as the coagulant. The protein coagulant may for example be an enriched blood plasma (containing a cell adhesion protein). Once again we believe that there is preferential deposition of the cell adhesion protein at the surface of the substrate. This procedure is particularly effective when the polysaccharide is chitosan.

In an alternative method of producing the substrate, a surface layer of a cell adhesion protein may be applied to a preformed polysaccharide. Application of the protein layer may be effected, for example, in a coating bath containing a solution of protein or by a technique such as spraying. Stabilisation of the surface layer may be by a carbodiimide.

Examples of polysaccharides which may be used for the substrate include alginates, chitosan, polylysine and other polyamino acids, cationic starches, cationic derivatives of other hydrocolloids, collagen, gelatine, low-methoxyl pectin, carrageenans, chondroitin sulphate, hyaluronic acid, carboxymethyl cellulose, carboxymethyl starch, carboxymethyl guar, cellulose sulphate, dextran sulphate, gellan, xanthan, and anionic derivatives of other hydrocolloids. We particularly prefer that the polysaccharide is comprised of an alginate material cross-linked with calcium ions as other di- or higher valent cation capable of cross-linking alginates.

Particularly preferred examples of cell growth substrates in accordance with the invention are in the form of fibres having a core (providing the basal layer) which consists of, or is rich in, the polysaccharide material and a surface at which the cell adhesion protein is provided.

Fibres in accordance with the invention may have a diameter of 10-1000 $\mu$ m, more preferably 40-150 $\mu$ m, even more preferably 40-100 $\mu$ m, and ideally 50-80 $\mu$ m. the fibres may be of any appropriate length.

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Such fibres may be produced by spinning a dope comprised of a solution of the polysaccharide into a coagulation bath causing precipitation of the fibres. The dope may also contain dissolved cell adhesion protein which is to form the surface layer with the spinning technique being such that there is preferential initial precipitation of polysaccharide in the coagulation bath followed by later precipitation of the cell adhesion protein which thus forms a protein rich outer layer of the fibre (this layer being integral with the core). The dope for use in this process may for example comprise (based on the total weight of the polysaccharide and cell adhesion protein) 60-95% (preferably about 90%) by weight of the polysaccharide and 5-40% (preferably about 10%) by weight of the cell adhesion protein. The fibre produced by such a process may have a core comprised of 50-80% by weight of the polysaccharide and an outer layer comprised of 50-80% by weight of the cell adhesion protein and 20-50% by weight of the polysaccharide.

In an alternative spinning method, fibres may be formed by a co-axial extrusion technique in which a solution of the cell adhesion protein is extruded co-axially around a (separate) solution of the polysaccharide, both solutions being spun into the same coagulation bath, whereby a fibre having a polysaccharide core and a surface layer of the cell adhesion protein is formed.

In an alternative process of producing the fibres, a dope comprised of a solution of the polysaccharide (but not the cell adhesion protein) may be spun into a coagulation bath and the fibre thus formed is treated with the cell adhesion protein. This treatment may be effected, for example, by providing the cell adhesion protein in the coagulation bath so that the protein is adsorbed as a surface layer onto the basal polysaccharide layer. It is however more preferred that the cell adhesion protein is applied in a bath downstream of the coagulation bath. The conditions in the protein bath may be such as to ensure formation of a stabilised coating of the protein layer is obtained.

Furthermore, for all embodiments of fibre formation, the cell adhesion protein should be concentrated at the fibre surface. If the fibre is produced by co-spinning a

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solution of the polysaccharide and cell adhesion protein the combination of relative molecular size, hydrophilic/hydrophobic balance and relative stability can be used to cause preferential precipitation. If the fibre is produced by a two-stage process then concentration of the protein at the surface may be achieved by the use of concentration of the polysaccharide and protein at each stage, first stage mixed polysaccharide and protein, second stage predominantly protein plus surface active agents and/or stabilisers.

Whichever method is used, the protein should be stabilised at the surface and, in fact, the lower the amount of protein the more important the stabilisation becomes. Stabilisation may be effected by ensuring that parts of the molecular chain of the protein are embedded in the bulk polysaccharide. In the case where the polysaccharide has been cross-linked by divalent cations, stabilisation of the protein may be by divalent cation bridges. When chitosan is used to form the core, carrier cation bridging will only occur within the protein species which will help to stabilise the protein at the surface.

More specific embodiments of producing fibres in accordance with the invention are described below.

In one such embodiment, a fibre is produced by ejecting an aqueous solution of sodium alginate through a spinneret into a coagulation bath containing  $\text{Ca}^{2+}$  ions. The fibre is then passed through a fibronectin solution (or mixed protein solution) in a coating bath (downstream of the coagulation bath) which is at a pH that will give fibronectin a net positive charge causing it to be capable of interacting with the alginic acid. The fibronectin can be further bound to the alginate by passing the fibre through a coagulation/stabilisation bath at a pH that favours fibronectin to become negatively charged thus favouring divalent cation bridging so as to stabilise the fibronectin on the polysaccharide. Alternatively, this bath may incorporate carbodiimide for effecting covalent bonding of the protein to the polysaccharide the coagulation/stabilisation bath may contain agents that modify either directly the

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interaction of the fibre with cells (for example through the nature of a counterion, e.g. Zn, Ag, Mn, Ce) or indirectly by influencing the surrounding environment by diffusion of an active molecular species, such as growth factors, aggregating agents, chemoattractants, surfactants, etc.

As an alternative to applying the fibronectin in a coating bath, it is possible to apply a fibronectin coating by spraying a fibronectin solution onto the fibre. Spraying provides a means of thin coating (i.e. only several molecules thick) and also a method of coating that will potentially produce a fibrillar form of the coating if the conditions of shear etc. are set correctly. These conditions may also be adjusted to give orientation of the fibril formed in relation to the substrate.

In a further embodiment of fibre production, fibres may be found in a single stage process by spinning a dope containing dissolved sodium alginate and fibronectin into a solution of calcium or other divalent ions (which provide the driving force for precipitation). The dope is formulated such that the fibronectin is preferentially precipitated at the surface of the fibre. The relative amounts of the calcium alginate to the fibronectin in the dope would preferably be of the order of at least 80 parts by weight alginate and at most 20 parts fibronectin.

In the process described in the preceding paragraph, the fibre would be produced under conditions that encourage the globular nature of the protein. This may be achieved by use of a pH or temperature (for the coagulation bath) that causes chains of the protein molecule to "roll-up" on themselves with a tendency to embed the ends of the chain in the fibre structure.

In an alternative fibre production the process, a polyelectrolyte such as chitosan would be mixed with the fibronectin solution and a fibre precipitated by spinning into a sodium hydroxide bath. The molecular weight of the chitosan would be chosen to encourage fibre formation.

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As an alternative to the process described in the previous paragraph it is possible to spin a dope comprising a solution of chitosan (as the polysaccharide) to form a fibre which may subsequently be coated with fibronectin. This coating (of fibronectin) would be formed by charge interaction directly between the charged chitosan side chains and the amino acid groups of the fibronectin as well as by cationic bridging.

For all methods of fibre production, it may be appropriate to subject the spun fibres to stretching, washing, and/or drying operations. In the case where a (separate) surface treatment of the cell adhesion protein is applied after formation of the basal polysaccharide layer, it may be appropriate to effect stretching and/or washing prior to the treatment with the cell adhesion protein.

Whilst fibres are the preferred form of the cell growth substrate in accordance with the invention, other forms are possible. Examples include sheets and strips which may be produced by forming (by a knife over roll or transfer coat or slot dye method) a thin film of a solution of the polysaccharide which is then precipitated in a coagulation bath. As in the case of fibre formation, the solution may also incorporate the cell adhesion protein to be preferentially deposited on coagulation at the surface of the polysaccharide. Alternatively the solution to be precipitated in the coagulation bath need not include the cell adhesion protein which may then be applied subsequently to the sheet or strip by spraying with a solution of the protein. In this case, the nature of the coating is determined by the concentration of the protein in solution, the velocity, orifice, size and direction of spray relative to the surface. Judicious adjustment of these parameters should produce undenatured but aligned molecules of active protein. The surface layer of the cell adhesion protein may be applied to the sheet by spraying with a solution of the protein. By spraying at high concentration and flow rate through a small orifice, protein denaturation, fibril formation and alignment can be obtained and if this is directed in parallel to a surface then this alignment will be maintained in the surface coat obtained molecular alignment of the protein will then be reflected in the alignment of cellular species grown on the substrate.

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Irrespective of the physical form (fibre, sheet etc) of the cell growth substrate of the invention and also irrespective of the manner in which the cell adhesion protein surface layer is incorporated therein, it is preferred that basal polysaccharide layer is formed by a spinning or extruding a solution of sodium alginate into a bath containing calcium ions. Preferred sodium alginate for use in such a technique have a Guluronic acid (G) content of at least 35% by weight and a Manauronic acid (M) content of at most 65% by weight. Preferably the G-content is 35-70% by weight and the M-content is 65-30% by weight. M preferably also the sodium alginate has a viscosity for a 1% solution (in water) of the sodium alginate of 30-300 cP, more preferably 40-100 cP. The alginate solution to be spun or extruded into the coagulation bath should generally have a total dissolved solids content of less than 10% by weight, more preferably in the range 5-7%. The amount of the cation (e.g. calcium) present in the coagulation bath (to effect precipitation of the alginate) is preferably less than 1% by weight.

For products in accordance with the invention produced by coagulation of a solution of an alginate, it is possible for the alginate solution (to be coagulated) to contain at least one additional polysaccharide to modify the properties of the alginate. The additional polysaccharide may, for example, be one having  $\text{COO}^-$  groups along the polysaccharide chains, for example pectin, carboxymethyl cellulose N-, O-carboxymethyl chitosan, carrageenan, xanthan or gellan. Alternatively or additionally the polysaccharide to the coagulated with the alginate may be one having  $\text{SO}_4^{2-}$  groups provided along the polysaccharide chain, e.g. chondroitin sulphate, dermatan sulphate, heparan sulphate or heparan. Uncharged polysaccharides may be used in conjunction with the alginate, e.g. acemannan. The additional polysaccharide may be one which improves the water absorbency of the alginate. Further disclosure of products obtained by coagulation of an alginate solution containing at least one other polysaccharide are given in WO-A-9610106 (Innovative Technologies Ltd), the disclosure of which is incorporated herein by reference.

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For all cell growth substrates in accordance with the invention, the surface layer of the cell adhesion protein may be continuous or discontinuous. Thus, for example, in the case of a fibre, the protein may be provided continuously along and around the fibre length or as periodic repeats (e.g. of predetermined length) along the fibre length and at least partially around the circumference of the fibre, or as "stripe" which does not extend completely around the circumference and which extends continuously or discontinuously along the fibre length. If the cell adhesion protein layer is discontinuous, parts of the surface of the cell growth substrate may (when used for cell growth) be positively interactive ("talking") and other parts passive ("silent") and other parts negatively interactive ("discouraging"). In cellular terms, this means that a positive surface encourages cell adhesion spreading, motility and growth whereas a passive surface ("silent") may have a low level of interaction.

Cell growth substrates in accordance with the invention may be used in a number of forms for various cell growth applications. Purely by way of example, substrates in the form of fibres may be formed into a structure, e.g. random matrices (e.g. non-woven felts and fleeces), orientated matrices (fibres having some relative alignment), knitted structures (e.g. knitted cloths), braided structures (e.g. braided thread), bundled structures, and carded slivers. One preferred structure comprises fibres in accordance with invention laid in parallel or randomly to each other and for preference bonded to a supporting layer, e.g. a polyurethane film. This supporting layer may be adhesively coated.

A further possibility is for fibres to be arranged in an amorphous gel.

A further possibility relates to fibres produced with a polysaccharide (e.g. alginate) cross-linked by a di- or higher-valent cation (e.g. calcium). Such fibres may (using the techniques disclosed in WO-A-9613285 (Innovative Technologies Ltd)) be admixed with an aqueous solution of a hydrogel precursor material whereby the cations from the fibres cross-link the precursor material resulting in the formation of a hydrogel in which the molecules of the hydrogel precursor are cross-linked by the di- or higher-valent cations donated by the fibres. The admixture may incorporate a plasticiser. Subsequently water may be removed from the hydrogel so as to provide a

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dehydrated form thereof containing the fibres as reinforcement. Such a product is eminently suitable for use on wound healing during which fibres will become exposed at the surface of the product to provide a substrate for cell growth. The hydrogel precursor may for example be sodium alginate and the plasticiser may for example be glycerol, polyethylene glycol, sorbitol or a PEO/PPO polymer.

Cell growth substrates in the form of strips or sheets may for example be rolled into tubes or other three dimensional structures.

As indicated above, cell growth substrates in accordance with the invention may be used in a range of cell growth applications. If cell alignment on the surface of the substrate is important then this may be imposed by the nature of the cell and its relationship to its surface. For example, cell alignment may be determined by the size of a fibre on which the cell is grown. If cell-long alignment either across or parallel to a particular axis of the substrate is required then this can be accomplished by either exposure of the surface to flow which will produce a wall shear stress parallel to the desired orientation or to axial strain which would tend to cause the cells to lie across the axis of stress and therefore across the axis of the surface.

A number of specific (but non-limiting) example of uses of cell growth substrates in accordance with the invention will now be given.

#### Wound Therapy

The substrates may be used in wound therapy. For this purpose, a cell-growth substrate (in accordance with the invention) in the form of a flat sheet or film may be preferred. The film or sheet material may incorporate an agent to be delivered to the wound.

Alternatively, parallel or random arrays of fibres with or without seeded cells may be placed on the wound either individually, in a bundled or fixed to a support which may be adhesively coated. An example of a suitable support is polymeric film material particularly a breathable film (e.g. high MVTR film). The film may be one having an MVTR when in contact with liquid water which is at least twice that when

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in contact with moisture vapour (but not liquid water). For example, the MVTR in contact with water vapour only may be 3000-5000 g m<sup>-2</sup> 24hr<sup>-1</sup> (as measured by ASTM E96B) and an MVTR in the presence of liquid water (as measured by ASTM E96BW) of 8000 to 10000 g m<sup>-2</sup> 24hr<sup>-1</sup>. The support may have apparatus to allow exudate transfer. Whether or not a support is used, the fibres applied to the wound may incorporate growth factors for delivery to surface cells or incorporate agents that will influence the surrounding environment, e.g. bactericides etc. Mixtures of fibres may be applied to the wound, e.g. any two of (i) fibres seeded with cells, (ii) unseeded fibres, and (iii) fibres containing an agent to be delivered to the wound.

#### Cultured Epidermal and Dermal Substitutes

Cell growth substrates in accordance with the invention may be cultured with single layers of epidermal keratinocytes or dermal fibroblasts (either of which may be of autologous or heterologous origin.) The substrate (with cultured cells) may be used alone or in combination with similarly cultured substrates. These substrates and cells may be used for the treatment of partial thickness wounds, e.g. donor sites and for treatment of ulcers.

#### Tissue Augmentation/Repair

Cell growth substrates in the form of continuous fibres can be positioned in relation to a damaged organ or structure. They may be placed either singularly or in bundles during invasive or non-invasive therapy.

Alternatively, cell growth substrates in the form of fibres may be provided as an injectable suspension. The suspension may be introduced into the body along a catheter guide system or the fibres may be formed at the site. As an alternative, it is possible to formulate a solution containing the polysaccharide, the other coagulant therefore with at least one of the solutions containing cell adhesion protein and to apply these solutions to a patient under conditions such that fibre formation occurs *in situ*, the fibre formed possibly being continuous.

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## Orthopaedic

Cell growth substrates in the form of fibres may be aligned parallel to tendons and seeded *in situ* with appropriate cells, chondrocytes, etc. Alternatively, fibres plus cell may be cultured in a laboratory and then delivered to the patient. For both embodiments, the fibres may contain, or be associated with fibres constructed with hyaluronic acid or other cartilage-derived substances.

### Vascular Graft

Cell growth substrates in the form of fibres may be knitted, woven or spun into tubes to encourage cell growth to form a blood conduit.

## Nerve Regeneration

Damaged nerves can be repaired using fibres to link the two (separated) ends of the nerve thus providing a path along which the new nerve can grow.

### Drug delivery

Cell growth substrates may incorporate active molecules located in the polysaccharide layer. These agents may be used to influence the fibre incorporation into the tissue. Alternatively the agent may provide a drug reservoir for the purposes topical or systemic therapy.

For all of the above embodiments of the invention, the cell adhesion protein may be replaced by a blood plasma component.

The invention is further illustrated with reference to the following non-linking Examples and the Figures of the accompanying drawings which shown the results of the Examples.

For the Examples, the following procedures were used.

## Cell Culture

1920 1921 1922 1923 1924 1925 1926 1927 1928 1929 1930 1931 1932 1933 1934 1935 1936 1937 1938 1939 1940 1941 1942 1943 1944 1945 1946 1947 1948 1949 1950 1951 1952 1953 1954 1955 1956 1957 1958 1959 1960 1961 1962 1963 1964 1965 1966 1967 1968 1969 1970 1971 1972 1973 1974 1975 1976 1977 1978 1979 1980 1981 1982 1983 1984 1985 1986 1987 1988 1989 1990 1991 1992 1993 1994 1995 1996 1997 1998 1999 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018 2019 2020 2021 2022 2023 2024 2025 2026 2027 2028 2029 2030 2031 2032 2033 2034 2035 2036 2037 2038 2039 2040 2041 2042 2043 2044 2045 2046 2047 2048 2049 2050 2051 2052 2053 2054 2055 2056 2057 2058 2059 2060 2061 2062 2063 2064 2065 2066 2067 2068 2069 2070 2071 2072 2073 2074 2075 2076 2077 2078 2079 2080 2081 2082 2083 2084 2085 2086 2087 2088 2089 2090 2091 2092 2093 2094 2095 2096 2097 2098 2099 2100 2101 2102 2103 2104 2105 2106 2107 2108 2109 2110 2111 2112 2113 2114 2115 2116 2117 2118 2119 2120 2121 2122 2123 2124 2125 2126 2127 2128 2129 2130 2131 2132 2133 2134 2135 2136 2137 2138 2139 2140 2141 2142 2143 2144 2145 2146 2147 2148 2149 2150 2151 2152 2153 2154 2155 2156 2157 2158 2159 2160 2161 2162 2163 2164 2165 2166 2167 2168 2169 2170 2171 2172 2173 2174 2175 2176 2177 2178 2179 2180 2181 2182 2183 2184 2185 2186 2187 2188 2189 2190 2191 2192 2193 2194 2195 2196 2197 2198 2199 2200 2201 2202 2203 2204 2205 2206 2207 2208 2209 2210 2211 2212 2213 2214 2215 2216 2217 2218 2219 2220 2221 2222 2223 2224 2225 2226 2227 2228 2229 2230 2231 2232 2233 2234 2235 2236 2237 2238 2239 2240 2241 2242 2243 2244 2245 2246 2247 2248 2249 2250 2251 2252 2253 2254 2255 2256 2257 2258 2259 2260 2261 2262 2263 2264 2265 2266 2267 2268 2269 2270 2271 2272 2273 2274 2275 2276 2277 2278 2279 2280 2281 2282 2283 2284 2285 2286 2287 2288 2289 2290 2291 2292 2293 2294 2295 2296 2297 2298 2299 2300 2301 2302 2303 2304 2305 2306 2307 2308 2309 2310 2311 2312 2313 2314 2315 2316 2317 2318 2319 2320 2321 2322 2323 2324 2325 2326 2327 2328 2329 2330 2331 2332 2333 2334 2335 2336 2337 2338 2339 2340 2341 2342 2343 2344 2345 2346 2347 2348 2349 2350 2351 2352 2353 2354 2355 2356 2357 2358 2359 2360 2361 2362 2363 2364 2365 2366 2367 2368 2369 2370 2371 2372 2373 2374 2375 2376 2377 2378 2379 2380 2381 2382 2383 2384 2385 2386 2387 2388 2389 2390 2391 2392 2393 2394 2395 2396 2397 2398 2399 2400 2401 2402 2403 2404 2405 2406 2407 2408 2409 2410 2411 2412 2413 2414 2415 2416 2417 2418 2419 2420 2421 2422 2423 2424 2425 2426 2427 2428 2429 2430 2431 2432 2433 2434 2435 2436 2437 2438 2439 2440 2441 2442 2443 2444 2445 2446 2447 2448 2449 2450 2451 2452 2453 2454 2455 2456 2457 2458 2459 2460 2461 2462 2463 2464 2465 2466 2467 2468 2469 2470 2471 2472 2473 2474 2475 2476 2477 2478 2479 2480 2481 2482 2483 2484 2485 2486 2487 2488 2489 2490 2491 2492 2493 2494 2495 2496 2497 2498 2499 2500 2501 2502 2503 2504 2505 2506 2507 2508 2509 2510 2511 2512 2513 2514 2515 2516 2517 2518 2519 2520 2521 2522 2523 2524 2525 2526 2527 2528 2529 2530 2531 2532 2533 2534 2535 2536 2537 2538 2539 2540 2541 2542 2543 2544 2545 2546 2547 2548 2549 2550 2551 2552 2553 2554 2555 2556 2557 2558 2559 2560 2561 2562 2563 2564 2565 2566 2567 2568 2569 2570 2571 2572 2573 2574 2575 2576 2577 2578 2579 2580 2581 2582 2583 2584 2585 2586 2587 2588 2589 2590 2591 2592 2593 2594 2595 2596 2597 2598 2599 2600 2601 2602 2603 2604 2605 2606 2607 2608 2609 2610 2611 2612 2613 2614 2615 2616 2617 2618 2619 2620 2621 2622 2623 2624 2625 2626 2627 2628 2629 2630 2631 2632 2633 2634 2635 2636 2637 2638 2639 2640 2641 2642 2643 2644 2645 2646 2647 2648 2649 2650 2651 2652 2653 2654 2655 2656 2657 2658 2659 2660 2661 2662 2663 2664 2665 2666 2667 2668 2669 2670 2671 2672 2673 2674 2675 2676 2677 2678 2679 2680 2681 2682 2683 2684 2685 2686 2687 2688 2689 2690 2691 2692 2693 2694 2695 2696 2697 2698 2699 2700 2701 2702 2703 2704 2705 2706 2707 2708 2709 2710 2711 2712 2713 2714 2715 2716 2717 2718 2719 2720 2721 2722 2723 2724 2725 2726 2727 2728 2729 2730 2731 2732 2733 2734 2735 2736 2737 2



L929 mouse fibroblast cells for use in an experiment were grown to confluence and then released from the tissue culture dishes by washing with Hepes Saline, followed by treatment with 0.25% trypsin solution. The resulting supernatant was centrifuged and the pellet of cells re-suspended in Dulbecco's modified Eagle's Medium [containing 10% Foetal calf serum, 5% Penicillin/Streptomycin, 1% ITS (Insulin transferrin selenite)]. If being sub-cultured, then the cells were plated out on tissue culture plates at a 1:5 dilution.

15mg of each fibre type to be tested were weighed out and placed in each well of a 12 well tissue culture dish. In all experiments the fibres were washed in serum containing media for a period of 24 hours. The experimental controls were cells plated on tissue culture plastic. Cells used for fibre testing were plated out at a density of 80,000 cells per well. All experiments were terminated up to a 72 hour timepoint.

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### Fixation and Staining

The cells and fibres were washed twice in Phosphate Buffered Saline (PBS) and then fixed using formalin solution (10% neutral buffered) for 10 minutes. The fixative was removed and the cells and fibres washed twice more in PBS. The cells were then stained with Geimsa for 10 minutes, followed by 3, five minute washes in PBS. The cells were then viewed using a Nikon Diaphot microscope and images captured using a JVC DVI digital camcorder. The images were then downloaded to an Apple Macintosh Power PC Performa 6400/200 and analysis performed using the public domain program NIH image. For the scanning electron microscopy, fixed samples were dehydrated in 100% ethanol for a period of 2 hours. The samples were then sputter coated using a Denton Vacuum desk 1. The samples were mounted on a stub and viewed using a Hitachi S-510 scanning electron microscope. Images are captured using the JVC camera and analysed on the Macintosh computer using NIH image.

### Preparation of Enriched Bovine Blood Plasma

Bovine blood was taken and mixed in a 9:1 ratio with a 4%w/w aqueous solution of trisodium citrate (Sigma Chemicals) as an anti-coagulant. The mixture was then centrifuged at 1000rpm for 10 minutes, after which time the supernatant plasma was pipetted off, frozen at  $-15$  to  $20^{\circ}\text{C}$  and then thawed under refrigeration at  $4^{\circ}\text{C}$ . This caused the globular protein content of the plasma to remain precipitated and become concentrated by sedimentation at the bottom of the storage vessel. The supernatant from the refrigerated plasma was removed and the remaining fraction when thawed formed a plasma further enriched in globular protein concentration which may be further enriched by another freeze thaw cycle. The plasma fraction thus isolated was used in some of the experiments outlined in the Examples below.

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### Example 1 (Comparative)

Calcium alginate fibres were produced by ejection at 12m/min of a 5.5%w/w aqueous solution of sodium alginate (ex Pronova Biopolymer, having a guluronic acid content of 70%) through a spinneret having 40,000 holes each of 70 $\mu$  diameter into a coagulation bath of calcium chloride dihydrate (1.5%w/w) and the resulting fibres were washed in acetone and dried. The fibres were observed under a scanning electron microscope (Hitachi model S510) and were found to be about 10-20 $\mu$  diameter smooth, cylindrical with few outstanding surface topographical features (see Figure 1). The cell adhesion properties of the fibres were then assessed using L929 mouse fibroblast cells according to the method outlined above. No cell attachment to the fibres was observed within 2 hours during which time the fibres formed a gel and then disintegrated.

### Example 2

A mixture of 5.5%w/w aqueous solution of sodium alginate (as in Example 1) with bovine blood plasma was prepared by mixing the components in a ratio of 3:2. This mixture was then used to produce fibres in the laboratory by ejection from a 1ml insulin syringe through a needle of 35 $\mu$  outside diameter into a coagulation bath of calcium chloride dihydrate (1.5%w/w) and the resulting fibres were washed in acetone and dried. Fibres were observed under the scanning electron microscope (see Figure 2a). The cell adhesion properties of the fibres were then assessed using L929 mouse fibroblast cells according to the method outlined above. Within 48 hours cells had grown to confluence on the fibres (see Figure 2b), a considerable improvement of the result observed in Example 1.

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Example 3 (Comparative)

A 3% w/w of chitosan, having a degree of de-acetylation >70% (available from Nigerian Fisheries), in 2% aqueous glacial acetic acid was prepared.

Chitosan fibres were made in the laboratory by ejecting the chitosan solution from a 1ml insulin syringe through a needle of 35 $\mu$  outside diameter into a coagulation bath of sodium hydroxide (5%w/w) and the resulting fibres were dried. The fibres were observed under the scanning electron microscope, the fibres were found to have a diameter of 40-100 $\mu$  and to be smooth, cylindrical with few outstanding surface topographical features. The cell adhesion properties of the fibres were then assessed using L929 mouse fibroblast cells according to the method outlined above. After 48 hours, a number of cells were found to adhere to the fibres but no evidence for cell elongation and alignment was apparent (see Figure 3).

Example 4

Chitosan fibres were made in the laboratory by ejecting a solution of chitosan (as specified in Example 3) from a 1ml insulin syringe through a needle of 35 $\mu$  outside diameter into a coagulation bath of enriched bovine blood plasma (isolated as described above) and the resulting fibres were washed in acetone and dried. The cell adhesion properties of the fibres were then assessed using L929 mouse fibroblast cells according to the method outlined above. Within 48 hours cells had grown to confluence (as seen from Figure 4) on the fibres, a far higher degree of cell attachment than that observed for fibres coagulated in sodium hydroxide (compare Example 3).

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### Claims

1. A substrate for cell growth comprising a polysaccharide and a cell adhesion protein provided at the surface of the substrate and non-covalently associated therewith.
2. A substrate as claimed in claim 1 comprising a polysaccharide basal layer having a surface layer incorporating the cell adhesion protein.
3. A substrate as claimed in claim 2 wherein the polysaccharide basal layer has a thickness greater than 60% of the thickness of this layer and the polysaccharide basal layer.
4. A substrate as claimed in claim 3 wherein the polysaccharide basal layer has a thickness greater than 80% of the thickness of this layer and the polysaccharide basal layer.
5. A substrate as claimed in any one of claims 2 to 4 wherein the cell adhesion protein layer is integral with the polysaccharide basal layer.
6. A substrate as claimed in claim 5 wherein the layer of the cell adhesion protein has a thickness of 1-20 $\mu$ m.
7. A substrate as claimed in any one of claims 2 to 5 wherein the layer of the cell adhesion protein is a surface adsorbed layer.
8. A substrate as claimed in claim 7 wherein the layer of the cell adhesion protein is 3-5 molecules deep.
9. A substrate as claimed in any one of claims 2 to 8 wherein the polysaccharide basal layer comprises at least 80% by weight of polysaccharide.
10. A substrate as claimed in claim 9 wherein the polysaccharide basal layer comprises at least 90% by weight of polysaccharide.

11. A substrate as claimed in any one of claims 2 to 10 wherein the surface layer of the cell adhesion protein comprises at least 80% by weight of cell adhesion protein.
12. A substrate as claimed in claim 11 wherein the surface layer of the cell adhesion protein comprises at least 90% by weight of cell adhesion protein.
13. A substrate as claimed in claim 12 wherein the surface layer of cell adhesion protein comprises 95 to 100% by weight of cell adhesion protein.
14. A substrate as claimed in any one of claims 2 to 13 wherein the cell adhesion protein layer is discontinuous layer.
15. A substrate as claimed in any one of claims 2 to 14 wherein the polysaccharide layer incorporates an active agent.
16. A substrate as claimed in claim 15 wherein the active agent is encapsulated.
17. A substrate as claimed in claim 15 or 16 wherein the active agent is a drug, growth factor or chemotactic agent.
18. A substrate as claimed in any one of claims 1 to 17 wherein the polysaccharide is selected from alginates, chitosan, polylysine and other polyamino acids, cationic starches, cationic derivatives of other hydrocolloids, collagen, gelatine, low-methoxyl pectin, carrageenans, chondroitin sulphate, hyaluronic acid, carboxymethyl cellulose, carboxymethyl starch, carboxymethyl guar, cellulose sulphate, dextran sulphate, gellan, xanthan, and anionic derivatives of other hydrocolloids.
19. A substrate as claimed in claim 18 wherein the polysaccharide is an alginate.
20. A substrate as claimed in claim 19 wherein the alginate has a Guluronic acid (G) content of at least 35% by weight and Mannuronic acid (M) content of at most 65% by weight.

21. A substrate as claimed in claim 20 wherein the polysaccharide has a G content of 35-70% by weight and an M content of 65-30% by weight.

22. A substrate as claimed in any one of claims 19 to 21 wherein the alginate is cross-linked with divalent cations, preferably calcium ions.

23. A substrate as claimed in claim 22 wherein the cell adhesion protein is stabilised by calcium ion bridges.

24. A substrate as claimed in claim 18 wherein the polysaccharide is chitosan.

25. A substrate as claimed in claim 24 wherein the chitosan comprises at least 70% de-acetylated chitin.

26. A substrate as claimed in any one of claims 1 to 25 wherein the cell adhesion protein is present in blood plasma.

27. A substrate as claimed in any one of claims 1 to 26 wherein the cell adhesion protein incorporates the RGD binding site.

28. A substrate as claimed in claim 27 wherein the cell adhesion protein is fibronectin, vitronectin or von Willebrand protein.

29. A substrate as claimed in claim 28 wherein the cell adhesion protein is fibronectin.

30. A substrate for cell growth comprising a polysaccharide and a blood plasma component provided at the surface of the substrate and non-covalently associated therewith.

31. A substrate as claimed in any one of claims 1 to 30 in the form of a fibre.

32. A substrate as claimed in claim 31 wherein the fibre has a diameter of 10-1000 $\mu$ m.
33. A substrate as claimed in claim 32 wherein the fibre has a diameter of 40-150 $\mu$ m.
34. A substrate as claimed in claim 33 wherein the fibre has a diameter of 40-100 $\mu$ m.
35. A substrate as claimed in claim 34 wherein the fibre has a diameter of 50-80 $\mu$ m.
36. A substrate as claimed in any one of claims 1 to 30 which is in the form of a sheet or film.
37. A substrate as claimed in claim 36 having a thickness of 2-2000 $\mu$ m.
38. A substrate as claimed in claim 37 having a thickness of 10-100 $\mu$ m.
39. A substrate as claimed in claim 37 having a thickness of 200-1000 $\mu$ m.
40. A substrate as claimed in claim 37 having a thickness of 500-2000 $\mu$ m.
41. An assembly of fibres as claimed in any one of claims 31 to 35.
42. An assembly as claimed in claim 41 in the form of a random matrix (e.g. a non-woven felt or fleece), orientated matrix (fibres having some relative alignment), a knitted structure, a braided structure, a bundled structure or a carded sliver.
43. An assembly comprising a plurality of fibres as claimed in any one of claims 31 or 35 wherein the fibres are arranged in parallel to each other.
44. An assembly comprising a plurality of fibres as claimed in any one of claims 31 or 34 wherein the fibres are arranged randomly.



45. An assembly as claimed in claim 43 or 44 wherein the fibres are provided on a support in the form of a sheet or film.

46. An assembly as claimed in claim 45 wherein the fibres are provided on a high MVTR film.

47. An assembly as claimed in claim 41 wherein said fibres are provided in a matrix of an amorphous gel.

48. A method of producing a cell growth substrate comprising extruding a solution containing dissolved polysaccharide and cell adhesion protein, the polysaccharide being present in an amount greater than the cell adhesion protein, into a coagulation bath such that there is precipitated a substrate comprised of a basal layer of a polysaccharide and a surface layer of cell adhesion protein non-covalently associated therewith.

49. A method as claimed in claim 48 wherein the substrate is precipitated in a bath containing divalent cations typically calcium ions.

50. A method as claimed in claim 48 or 49 wherein the solution to be extruded comprises (based on the total weight of the polysaccharide and cell adhesion protein) 60-99% by weight of the polysaccharide and 1-40% by weight of the cell adhesion protein.

51. A method as claimed in claim 49 or 50 wherein the dissolved polysaccharide is sodium alginate.

52. A method as claimed in claim 51 wherein the sodium alginate has a G-content of 35-70% by weight and an M-content of 65-35% by weight.

53. A method as claimed in claim 51 or 52 wherein the sodium alginate has a viscosity for a 1% solution (in water) of 30-300 cP.
54. A method as claimed in claim 53 wherein the sodium alginate has a viscosity of a 1% solution (in water) of 40-100 cP.
55. A method of producing a cell growth substrate comprising applying to the surface of a preformed layer of a polysaccharide a surface layer of a cell adhesion protein or blood plasma component such that said cell adhesion protein or blood plasma component is non-covalently associated with the polysaccharide.
56. A method as claimed in claim 55 wherein the method of application is by immersion of the polysaccharide layer in a coating bath containing the cell adhesion protein or blood plasma component.
57. A method as claimed in claim 55 wherein the polysaccharide is precipitated into a coagulation bath containing the cell adhesion protein.
58. A method as claimed in claim 55 wherein the method of application is by spraying.
59. A method as claimed in any one of claims 55 to 58 effected with stabilisation of the protein layer.
60. A method of cell culture comprising effecting growth of cells on a substrate as claimed in any one of claims 1 to 40 or an assembly as claimed in any one of claims 41 to 47.
61. A method as claimed in 60 wherein the cell growth is for wound repair, tissue repair or augmentation, culturing epidermal or dermal substitute, orthopaedic application, vascular grafts, nerve regeneration or cell culture in a bioreactor.

62. The use of a substrate as claimed in any one of claims 1 to 40 or an assembly as claimed in any one of claims 41 to 47 in therapy.

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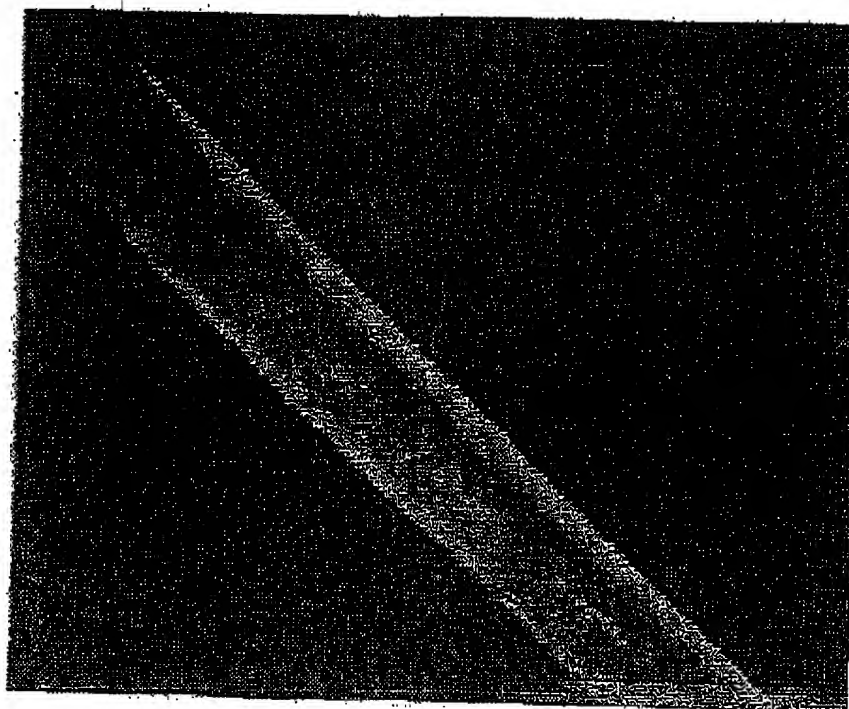


FIG. 1

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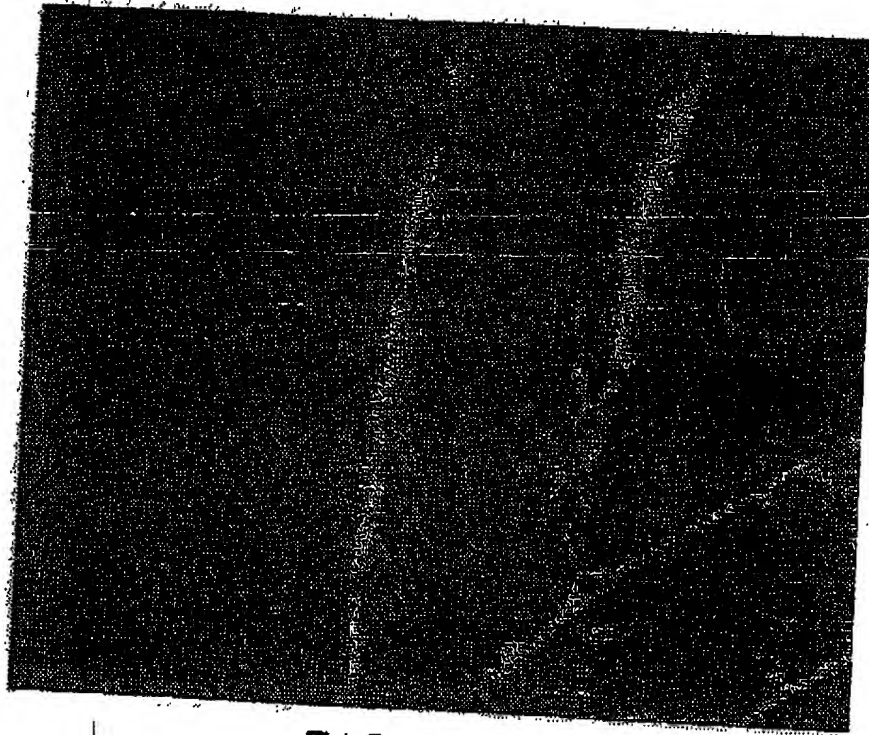


FIG. 2a

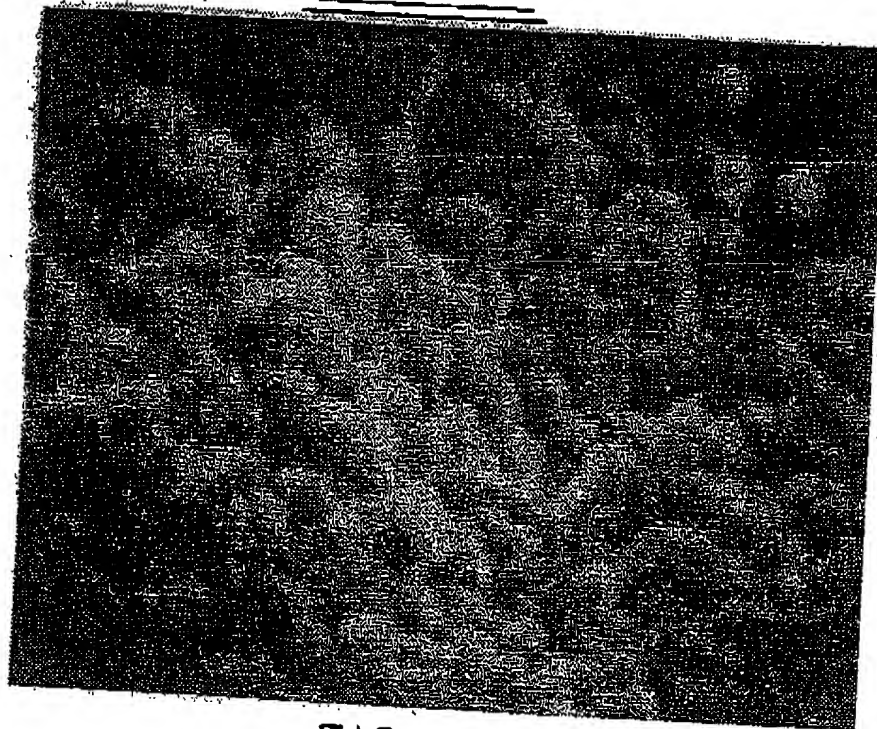


FIG. 2b

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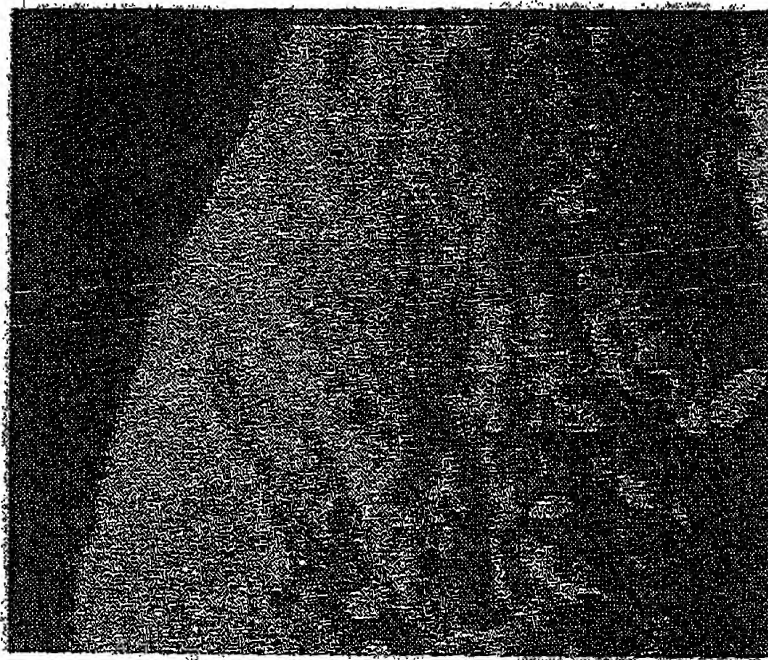


FIG. 3



FIG. 4

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# DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

135471; WENMM SB/01 (2-98)

Attorney Docket Number 7250-12  
First Named Inventor Douglas William Hamilton

## COMPLETE IF KNOWN

Application No. 09/889,715  
Filing Date  
Group An Unit  
Examiner's Name

☒ Declaration submitted with Initial Filing

☒ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16(e)) required)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

### CELL GROWTH

the specification of which (check one)

☐ is attached hereto.

☒ Was filed on January 21, 2000 as United States Application No. or

PCT International Application No. PCT/GB00/00145

☐ And was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

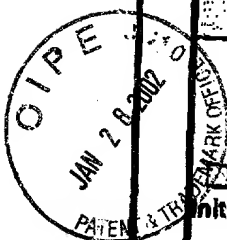
I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YY)	Priority Not Claimed	Certified Copy Attached?	
GB00/00145	PCT	01/21/00		Yes	No
GB 9901272.6	GB	01/21/99		<input type="checkbox"/>	<input checked="" type="checkbox"/>
GB 9903561.0	GB	02/17/99		<input type="checkbox"/>	<input checked="" type="checkbox"/>

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.



0989715-01202

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

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As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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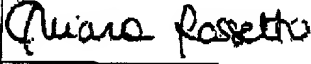
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are true to the best of my knowledge and belief and that I am not making any statement which I know to be false or which I believe to be false and that such willful false statements may jeopardize the validity of the application.

*Doug*  
*Please Sign here*  
↓

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Inventor's Signature:		Date of Signature:	
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WENMM SB/02C (3-97)

# DECLARATION

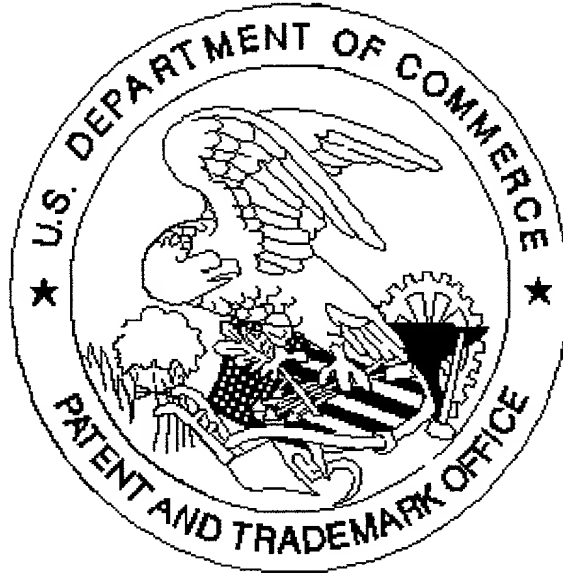
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